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J-15-34/35 GENETIC CONSTRUCTION REPORT

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THIS REPORT CONTAINS TSCA CONFIDENTIAL BUSINESS INFORMATION

SUBMITTER: MASCOMA LLC

I. INTRODUCTION

Mascoma LLC has constructed two *Saccharomyces cerevisiae* strains [M11321 & M11807]. The recipient strain is *S. cerevisiae* M2390, and it was isolated from a commercial sample of yeast used for ethanol production. Modifications to this parental strain have been the subject of MCAN J-13-3 through J-13-06, and J-14-16, which were all dropped from review.

II. INTENDED USE

100% of the microorganisms will go to cellulosic fuel ethanol production. The strains are intended to be used in place of traditional yeast which is unable to ferment xylose.

III. DESCRIPTION OF GENETIC MODIFICATION

Parent or Recipient strain: *S. cerevisiae* M2390

The recipient strain is an intergeneric microorganism that was modified through multiple linear DNA transformations using various plasmids in order to produce the two new strains submitted. Both strains now express sixteen copies of the codon-optimized xylose isomerase (XI) (EC #5.3.1.5) taken from *Catonella morbi*, additional copies of the *S. cereviseae* genes XKS1, TAL1, TKL1, RPE1, and RKI1, but only one strain (M11807) also expresses four copies of the bifunctional acetaldehyde-CoA/alchohol dehydrogenase (adhE) (EC #1.2.1.10 & 1.1.1.1) from *Bifidobacterium adolescentis*, plus native genes STL1 (to increase uptake of glycerol to reduce the extracellular concentration of glycerol) and ACS2 (to increase conversion of acetate to acetyl-CoA). The open reading frames of three native genes, GRE3, YPR1 and GPD2, were deleted and a single amino acid change was engineered into the native gene YFH1 in both strains.

The deletion of FCY1 eliminates *S. cerevisiae*'s ability to use cytosine as a nitrogen source and thus cannot grow on media where cytosine is the sole source of nitrogen. This modification allows the new strains to be easily distinguished from wild-type strains.

The following **intergeneric genes** were modified in the final strains:

Strain	Gene	Source Organism	Function	Artificially Synthesized
M11321& M11807	xylose isomerase (XI; xylA; EC 5.3.1.5)	*Catonella morbis*	Converts D-xylose to D-xylulose & D-glucose to D-Fructose	Yes
M11807 only	acetaldehyde-CoA/alchohol dehydrogenase (adhE; EC 1.2.1.1 & 1.1.1.1)	*Bifidobacterium adolescentis*	Converts acetyl-CoA to acetaldehyde	Yes

Genetic modifications contained in M11321 and M11807 (arrows = intergeneric genes)

Strain	Locus	Locus Modification	Cassette	Co-transformation	Cassette Description
M2390					
M3250	GRE3; aldose reductase	K(N)T-MX cassette	MA629		marked deletion of GRE3
M8555	GRE3; aldose reductase	replaced with enzyme cassette	MA1043	pMU228	2 copies of XKS1/TAL1/TKL1/RKI1/RPE1
M8671	FCY1; cytosine deaminase	replaced with enzyme cassette	MA1238	pMU228	deletion of FCY1; 4 copies CX355 XI
M8678	YLR296W; dubious open reading frame	K(N)T-MX cassette	MA1067		marked deletion of YLR296W
M8793	YLR296W; dubious open reading frame	replaced with enzyme cassette	MA1250	pMU228	4 copies CX355 XI
M9027	YFH1; mitochondrial matrix iron chaperone	CYC1t-K(N)T-MX cassette	MA1502		marked insertion of CYC1t at YFH1
M9113	YFH1; mitochondrial matrix iron chaperone	replaced with YFH1^{T163P}	MA1343	pMU228	amino acid substitution (T163P) in YFH1
M9231	APT2; adenine phosphoribosyltransferase	K(N)T-MX cassette	MA959		marked deletion of APT2
M9325	APT2; adenine phosphoribosyltransferase	replaced with enzyme cassette	MA1579	pMU228	4 copies CX355 XI
M9710					
M9906	YPR1; aldo-keto reductase	replaced with enzyme cassette	MAP93	pMU3993, pMU3974	2 copies of XKS
M9913	IME1; master regulator of meiosis	K(N)T-MX cassette	MA1171		marked deletion of IME1
M9944	IME1; master regulator of meiosis	replaced with enzyme cassette	MA1564	pMU228	4 copies CX355 XI
M10690	FCY1, YLR296W, APT2, IME1	replaced CX355 with GR2 and GR3	MA1594, 1595, 1799, 1800	pMU3993, pMU3940	deletion of CX355 cassettes with new gRNA target sequences GR2, GR3
M11205	FCY1, YLR296W	replaced GR2 with enzyme cassette	MA1891, MA1803	pMU3993, pMU3934	4 copies of Cm XI at each locus
M11321	APT2, IME1	replaced GR3 with enzyme cassette	MA1801, MA1802	pMU3993, pMU3935	4 copies of Cm XI at each locus
M11807	GPD2; NAD-dependent glycerol 3-phosphate dehydrogenase	replaced with enzyme cassette	MAP665	pMU3993, pMU3948	4 copies of adhE; 2 copies of ACS2/STL1

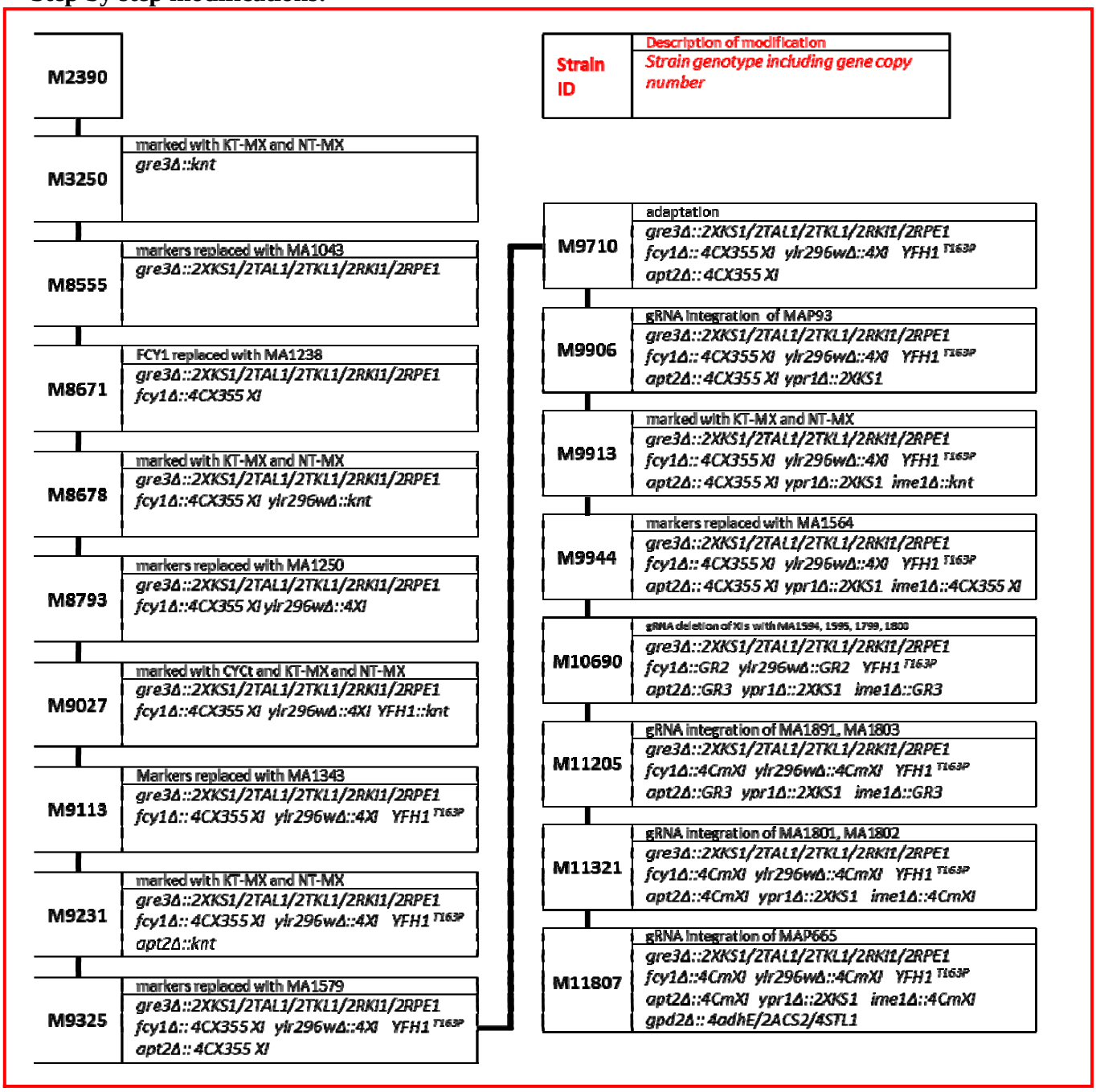
Genes integrated into strain M11321 (arrows = intergeneric genes)

Gene	Enzyme	EC number	Organism	Modification	Promoter	Terminator
XI	xylose isomerase	5.3.1.5	*C. morbi*	8 copy integration	HSP150	DIT1
XI	xylose isomerase	5.3.1.5	*C. morbi*	8 copy integration	ADH1	YHI9
XKS1	xylulokinase	2.7.1.17	*S. cerevisiae*	4 copy integration	GPM1	TEF2
TAL1	transaldolase	2.2.1.2	*S. cerevisiae*	2 copy integration	TEF2	SOL1
TKL1	transketolase	2.2.1.1	*S. cerevisiae*	2 copy integration	CCW12	FBA1
RPE1	ribulose-5-phosphate epimerase	5.1.3.1	*S. cerevisiae*	2 copy integration	HXT7	TDH3
RKI1	ribulose-5-phosphate isomerase	5.3.1.6	*S. cerevisiae*	2 copy integration	FBA1	GPM1

Genes integrated into strain M11807 (arrows = intergeneric genes)

Gene	Enzyme	EC / TC number	Organism	Modification	Promoter	Terminator
XI	xylose isomerase	5.3.1.5	*C. morbi*	8 copy integration	HSP150	DIT1
XI	xylose isomerase	5.3.1.5	*C. morbi*	8 copy integration	ADH1	YHI9
adhE	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	1.2.1.10 & 1.1.1.1	*B. adolescentis*	2 copy integration	GPD2	HXT2
adhE	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	1.2.1.10 & 1.1.1.1	*B. adolescentis*	2 copy integration	TPI1	FBA1
XKS1	xylulokinase	2.7.1.17	*S. cerevisiae*	4 copy integration	GPM1	TEF2
TAL1	transaldolase	2.2.1.2	*S. cerevisiae*	2 copy integration	TEF2	SOL1
TKL1	transketolase	2.2.1.1	*S. cerevisiae*	2 copy integration	CCW12	FBA1
RPE1	ribulose-5-phosphate epimerase	5.1.3.1	*S. cerevisiae*	2 copy integration	HXT7	TDH3
RKI1	ribulose-5-phosphate isomerase	5.3.1.6	*S. cerevisiae*	2 copy integration	FBA1	GPM1
ACS2	acetyl-coA synthetase	6.2.1.1	*S. cerevisiae*	2 copy integration	PYK1	ENO1
STL1	glycerol proton symporter	2.A.1.1.38	*S. cerevisiae*	2 copy integration	TEF2	ADH3
STL1	glycerol proton symporter	2.A.1.1.38	*S. cerevisiae*	2 copy integration	ADH1	PDC1

Step by step modifications:



Inserted Sequence Verification

All of the modified loci in the final strains were sequenced verified.

Possibility of Genetic Transfer

The submitter states that the transfer capability of the production strains is low because certain key elements that lead to or improve the potential for transfer are noticeably absent from the constructs. As an initial matter, the production strains do not contain any extra chromosomal elements, such as plasmids.

Antibiotic Resistance (ABR) Markers

Although plasmids and integrated DNA used during the construction of these strains contained antibiotic resistance markers for hygromycin (HYG), G418 (KAN), nourseothricin (NAT), and ampicillin (Amp); **NO** antibiotic resistance genes are present in the new strains. PCR analysis is provided to show that ABR markers are not present. In addition growth assays in the presence of antibiotics demonstrate that the strains are unable to grow in and are sensitive to antibiotic containing growth media.

In addition the host strain M2390 is known to be sensitive to the common antifungals amphotericin B and Fluconazole, and as no antifungal selection markers were used, the new strains are believed to retain this antifungal susceptibility.

IV. BY-PRODUCTS

Yeast Production

The expected byproducts are spent nutrient broth, ethanol, and carbon dioxide.

Cellulosic Fuel Ethanol Production

The major byproducts are carbon dioxide, process water and process solids. Carbon dioxide is vented to the atmosphere or may be recovered as a purified product. The process water can be recirculated and reused back into the ethanol process. The process solids, which consist mostly of inactivated biomass, such as lignin, may be recovered to be used as a fuel source through processes such as lignin gasification. Minor byproducts of cellulosic fuel ethanol production include: plant oils, glycerol, lactic acid and acetic acid.

V. PRODUCTION VOLUME

Year	Weight (tons)	Concentration (CFU/g)	Total CFUs
1	22	$5.76x10^{9}$	$1.27x10^{11}$
2	NA	NA	NA
3	55	$5.76x10^{9}$	$3.2x10^{11}$

The MCAN strains will be manufactured as a Stabilized Liquid Yeast (SLY) or yeast cream.

In the 1st year, 22 tons of yeast cream will be manufactured with an expected concentration of $5.76x10^{9}$ CFU/g; resulting in $1.27x10^{11}$ CFUs.

In the 3rd year, anticipated production volume is 55 tons of yeast cream; resulting in $3.2x10^{11}$ CFUs.